Journal of Clinical Microbiology, Jan. 2010, p. 208–214 Vol. 48, No. 1
0095-1137/10/$12.00 doi:10.1128/JCM.01750-09

Internal Transcribed Spacer Region Sequence Heterogeneity in *Rhizopus microsporus*: Implications for Molecular Diagnosis in Clinical Microbiology Laboratories[▽]

Patrick C. Y. Woo,[1,2,3,4]* Shui-Yee Leung,[3] Kelvin K. W. To,[3] Jasper F. W. Chan,[3] Antonio H. Y. Ngan,[3] Vincent C. C. Cheng,[3] Susanna K. P. Lau,[1,2,3,4]* and Kwok-Yung Yuen[1,2,3,4]

State Key Laboratory of Emerging Infectious Diseases,[1] Research Centre of Infection and Immunology,[2] Department of Microbiology,[3] and Carol Yu Centre for Infection,[4] The University of Hong Kong, Hong Kong

Received 5 September 2009/Returned for modification 23 October 2009/Accepted 6 November 2009

Although internal transcribed spacer region (ITS) sequence heterogeneity has been reported in a few fungal species, it has very rarely been reported in pathogenic fungi and has never been described in *Mucorales*, causes of the highly fatal mucormycosis. In a recent outbreak investigation of intestinal mucormycosis due to *Rhizopus microsporus* infection in patients with hematological malignancies, PCR of the ITS of four of the 28 *R. microsporus* strains, P11, P12, D3-1, and D4-1, showed thick bands at about 700 bp. Direct sequencing of the purified bands showed frequent double peaks along all of the sequence traces and occasional triple peaks for P12, D3-1, and D4-1. The thick bands of the four *R. microsporus* strains were purified and cloned. Sequencing of 10 clones for each strain revealed two different ITS sequences for P11 and three different ITS sequences for P12, D3-1, and D4-1. Variations in ITS sequence among the different ribosomal DNA (rDNA) operons in the same strain were observed in only ITS1 and ITS2 and not the 5.8S rDNA region. One copy of P11, P12, and D4-1, respectively, and one copy of P11, P12, D3-1, and D4-1, respectively, showed identical sequences. This represents the first evidence of ITS sequence heterogeneity in *Mucorales*. ITS sequence heterogeneity is an obstacle to molecular identification and genotyping of fungi in clinical microbiology laboratories. When thick bands and double peaks are observed during PCR sequencing of a gene target, such a strain should be sent to reference laboratories proficient in molecular technologies for further identification and/or genotyping.

Genes and intergenic regions of ribosomal DNA (rDNA) operons are the most widely used targets for molecular identification of bacteria and fungi in clinical microbiology laboratories. For bacterial identification, the 16S rDNA gene is the primary target to amplify and sequence (28), whereas for fungi, the 18S rDNA gene and internal transcribed spacer region (ITS) comprising the ITS1-5.8S-ITS2 rDNA gene cluster are commonly used, depending on the group of fungi being identified (4, 10, 23, 27). Irrespective of the target, such a molecular identification technique usually involves PCR amplification of the target and purification and direct sequencing of the PCR product. Since most bacterial and fungal genomes contain more than one rDNA operon, the success of using this technology relies on sequence homogeneity in the various copies of targets in the rDNA operons within the genome of the bacterium or fungus.

Interoperon heterogeneities for 16S rDNA genes have been reported in a number of bacteria (3, 12). Recently, we reported rDNA operon heterogeneity in a novel genus and species of bacterium, *Anaerospora hongkongensis*, isolated from an intravenous drug user (25). When present, such rDNA operon heterogeneity will pose difficulties for direct sequencing of the PCR product for bacterial identification as double or multiple nucleotide peaks will be present in the sequence traces. Although ITS sequence heterogeneity has been reported in a few fungal species (13, 15, 22), it has very rarely been reported in pathogenic fungi and has never been described in members of the order *Mucorales*, the etiological agents of the highly fatal mucormycosis (1, 18, 19). Recently, during the outbreak investigation of intestinal mucormycosis due to *Rhizopus microsporus* in patients with hematological malignancies, 28 strains of *R. microsporus* were subjected to ITS sequencing (5). Direct

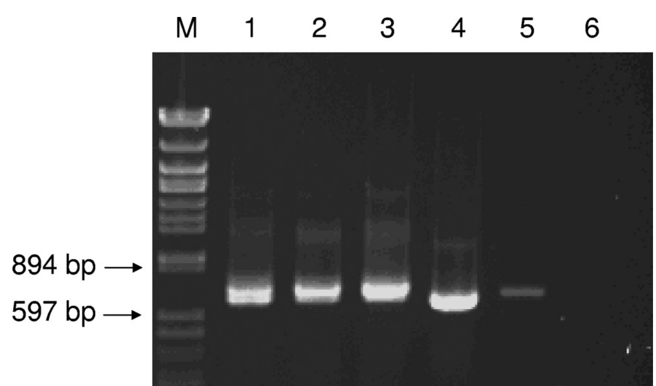

FIG. 1. DNA products from PCR of ITS in *R. microsporus*. Lane M, molecular marker Lambda AvaII digest; lane 1, strain P11; lane 2, strain P12; lane 3; strain D3-1; lane 4, strain D4-1; lane 5, strain P2 (positive control); lane 6, negative control containing DNase I-treated distilled water.

* Corresponding author. Mailing address: Department of Microbiology, The University of Hong Kong, University Pathology Building, Queen Mary Hospital, Hong Kong. Phone: (852) 28554892. Fax: (852) 28551241. E-mail for P. C. Y. Woo: pcywoo@hkucc.hku.hk. E-mail for S. K. P. Lau: skplau@hkucc.hku.hk.
▽ Published ahead of print on 11 November 2009.

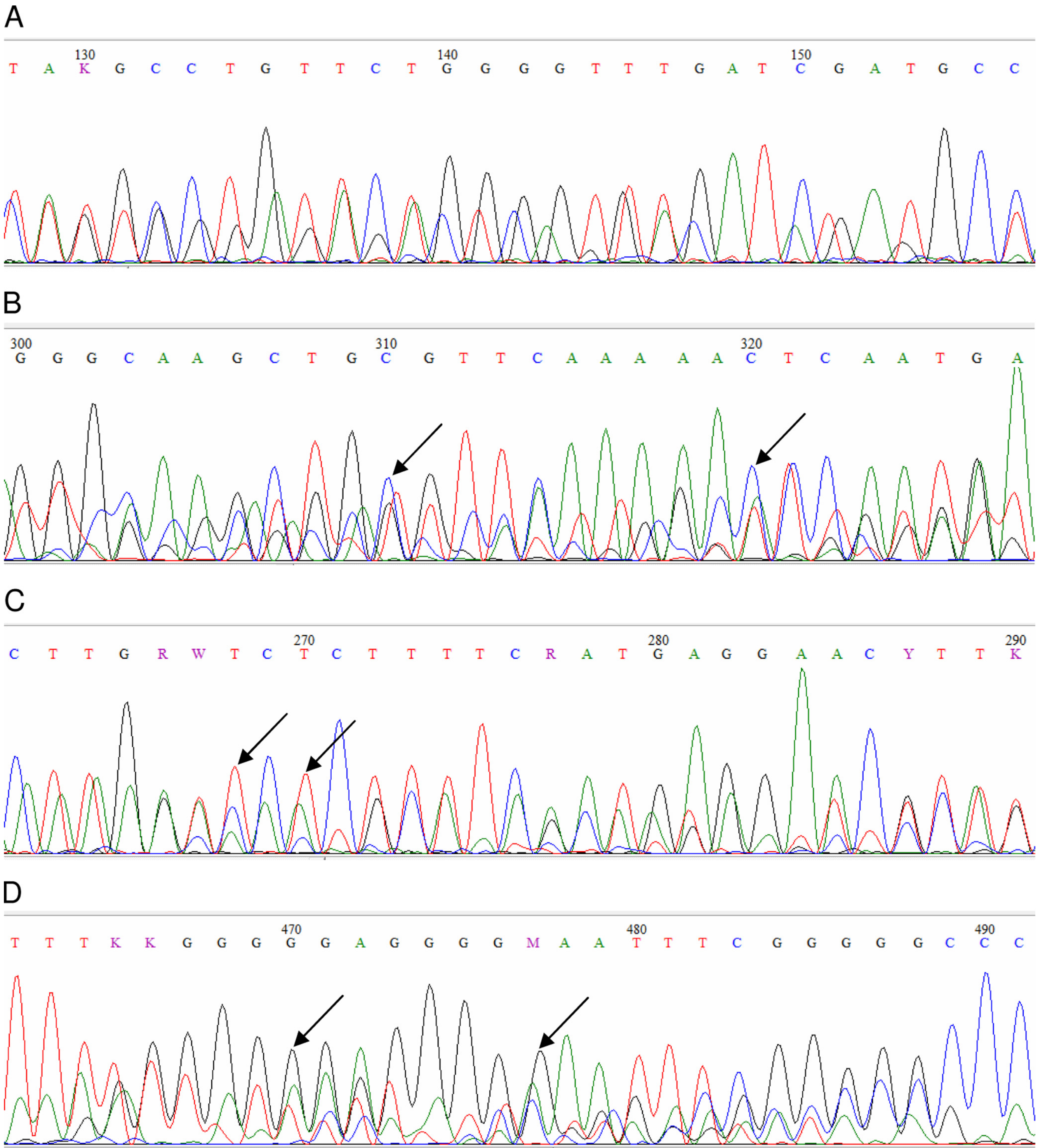

FIG. 2. Sequence traces from direct sequencing of the purified bands of *R. microsporus* shown in Fig. 1: strain P11 (A), strain P12 (B), strain D3-1 (C), and strain D4-1(D). Examples of triple peaks in strains P12, D3-1, and D4-1 are indicated by arrows.

sequencing of the PCR products from the 28 strains showed unambiguous sequence in 24 of them (5). For the other four strains, double peaks were observed frequently in the sequence traces. We hypothesize that these four strains possess ITS sequence heterogeneity. To test this hypothesis, we cloned the PCR products of these four strains and sequenced 10 clones from each strain. In this article, we report this phenomenon of ITS sequence heterogeneity in *R. microsporus*. The implications for molecular diagnosis in clinical microbiology laboratories are also discussed.

```
A
              18S rRNA                       ITS1
P11 copy 1  TCCGTAGGTG AACCTGCGGA AGGATCATTA ACTAAATGTA TCGGCACTTT ACTGGGAGAG  60
P11 copy 2  TCCGTAGGTG AACCTGCGGA AGGATCATTA ACTAA-TGTA TTGGCACTTT ACTGGGA---  56

P11 copy 1  GGGGGGATTC ATCCTCTCCT CCTGGTATTG TTTGCTCCTA TACTGTGAAT CTCTGGCGAT  120
P11 copy 2  ---------- ---TTTACTT CTCAGTATTG TTTGCTC-TA TACTGTGAAC CTCTGGCGAT  102

P11 copy 1  GAAGGTTTCG GTTGTTGTTA TGAATGATGA CTGAACCTTT GGGAGAGACT CAGGACATAT  180
P11 copy 2  GAAGGTC--- ---------- ------GTAA CTGA-CCTTC GGGAGAGACT CAGGACATAT  142

P11 copy 1  AGGCTATAAT GGGTAGGCCT GTTCTGGGGT TTGATCGATG CCAATCAGGT GTGCCTTGTG  240
P11 copy 2  AGGCTATAAT GGGTAGGCCT GTTCTGGGGT TTGATCGATG CCAATCAGGA TTACCTTTCT  202

P11 copy 1  TAC------- --------AC CTGGTACCCT TTGCCATATA CTATGAATTC AGGAATTTAA  285
P11 copy 2  TCCTTTGGGA AGGAAGGTGC CTGGTACCCT TTACCATATA CCATGAATTC AGAATT---G  259
                                5.8S rRNA
P11 copy 1  AAAGTTTAAA AAAAAAAACA ACTTTTAACA ATGGATCTCT TGGTTCTCGC ATCGATGAAG  345
P11 copy 2  AAAGTATAAT ATAATAA-CA ACTTTTAACA ATGGATCTCT TGGTTCTCGC ATCGATGAAG  318

P11 copy 1  AACGTAGCAA AGTGCGATAA CTAGTGTGAA TTGCATATTC GTGAATCATC GAGTCTTTGA  405
P11 copy 2  AACGTAGCAA AGTGCGATAA CTAGTGTGAA TTGCATATTC GTGAATCATC GAGTCTTTGA  378
                                                                  ITS2
P11 copy 1  ACGCAGCTTG CACTCTATGG ATCTTCTATA GAGTACGCTT GCTTCAGTAT CATAACAAAC  465
P11 copy 2  ACGCAGCTTG CACTCTATGG ATCTTCTATA GAGTACGCTT GCTTCAGTAT CATAACCAAC  438

P11 copy 1  CCACACATAA AAATCTTTTT TTTTTTTATG TGGTGATGGG CAAGTTCGGC GGCCCAGTAA  525
P11 copy 2  CCACACATAA AA-------T TTATTTTATG TGGTGATGGA CAAGCTCGGT TA------AA  485

P11 copy 1  TATTATTATT GCTTGCTGCT GATTGCCTAA AATACAGCC- TCTTTGTAAT TCTCGCATCG  584
P11 copy 2  TTTAATTATT A-----TACC GATTGTCTAA AATACAGCCC TCTTTGTAAT TTT--CATTA  538

P11 copy 1  AATTACGAAC TACCTAGCCA TCGTGCCTTT TTTGGTCCAA AAAACCAAAA AAACGTAAAA  644
P11 copy 2  AATTACGAAC TACCTAGCCA TCGTGCTTTT TTTGGTCCAA ----CCAAAA AAACATATAA  594

P11 copy 1  CCTAGGGGGG TTCTGCCAGC CAGCAGATAT TTGCATGCCT TCTCAACTAT GATCTGAAGT  704
P11 copy 2  TCTAGGG--G TTCTGCTAGC CAGCAGATAT TTTAATGATC TTT-AACTAT GATCTGAAGT  651
                       28S rRNA
P11 copy 1  CAAGTGGGAC TACCCGCTGA ACTT 728
P11 copy 2  CAAGTGGGAC TACCCGCTGA ACTT 675

B
              18S rRNA                       ITS1
P12 copy 1  TCCGTAGGTG AACCTGCGGA AGGATCATTA ACTAA-TGTA TTGGCACTTT ACTGGGA---  56
P12 copy 2  TCCGTAGGTG AACCTGCGGA AGGATCATTA ACTAA-TGTA TTGGCACTTT ACTGGGA---  56
P12 copy 3  TCCGTAGGTG AACCTGCGGA AGGATCATTA ACTAAATGTA TCGGCACTTT ACTGGGAGAG  60

P12 copy 1  ---------- ---TTTACTT CTCAGTATTG TTTGCT-CTA TACTGTGAAC CTCTGGCGAT  102
P12 copy 2  ---------- ---TTTACTT CTCAGTATTG TTTGCTTCTA TACTGTGAAC CTCTGGCGAT  103
P12 copy 3  GGGGGGATTC ATCCTCTCCT CCTGGTATTG TTTGCTCCTA TACTGTGAAT CTCTGGCGAT  120

P12 copy 1  GAAGGTC--- ---------- ------GTAA CTGA-CCTTC GGGAGAGACT CAGGACATAT  142
P12 copy 2  GAAGGTC--- ---------- ------GTAA CTGA-CCTTC GGGAGAGACT CAGGACATAT  143
P12 copy 3  GAAGGTTTCG GTTGTTGTTA TGAATGATGA CTGAACCTTT GGGAGAGACT CAGGACATAT  180

P12 copy 1  AGGCTATAAT GGGTAGGCCT GTTCTGGGGT TTGATCGATG CCAATCAGGA TTACCTTTCT  202
P12 copy 2  AGGCTATAAT GGGTAGGCCT GTTCTGGGGT TTGATCGATG CCAATCAGGA TTACCTTTCT  203
P12 copy 3  AGGCTATAAT GGGTAGGCCT GTTCTGGGGT TTGATCGATG CCAATCAGGT GTGCCTT---  237

P12 copy 1  TCCTTTGGGA AGGAAGGTGC CTGGTACCCT TTACCATATA CCATGAATTC AG-AATTGAA  261
P12 copy 2  TCCTTTGGGA AGGAAGGCGC CTGGTACCCT TTACCATATA TCATGAATTC AG-AATTGAA  262
P12 copy 3  ---------- --GTGTACAC CTGGTACCCT TTGCCATATA CTATGAATTC AGGAATTTAA  285
                                5.8S rRNA
P12 copy 1  AGTAT---AA TATAATAACA ACTTTTAACA ATGGATCTCT TGGTTCTCGC ATCGATGAAG  318
P12 copy 2  AGTAT---AA TATAATAACA ACTTTTAACA ATGGATCTCT TGGTTCTCGC ATCGATGAAG  319
P12 copy 3  AAAGTTTAAA AAAAAAAACA ACTTTTAACA ATGGATCTCT TGGTTCTCGC ATCGATGAAG  345

P12 copy 1  AACGTAGCAA AGTGCGATAA CTAGTGTGAA TTGCATATTC GTGAATCATC GAGTCTTTGA  378
P12 copy 2  AACGTAGCAA AGTGCGATAA CTAGTGTGAA TTGCATATTC GTGAATCATC GAGTCTTTGA  379
P12 copy 3  AACGTAGCAA AGTGCGATAA CTAGTGTGAA TTGCATATTC GTGAATCATC GAGTCTTTGA  405
                                                                  ITS2
P12 copy 1  ACGCAGCTTG CACTCTATGG ATCTTCTATA GAGTACGCTT GCTTCAGTAT CATAACCAAC  438
P12 copy 2  ACGCAGCTTG CACTCTATGG ATCTTCTATA GAGTACGCTT GCTTCAGTAT CATAACCAAC  439
P12 copy 3  ACGCAGCTTG CACTCTATGG ATCTTCTATA GAGTACGCTT GCTTCAGTAT CATAACAAAC  465

P12 copy 1  CCACACATAA AA-------T TTATTTTATG TGGTGATGGA CAAGCTCGGT TA------AA  485
P12 copy 2  CCACACATAA AA-------T TTATTTTATG TGGTGATGGA CAAATTCGGT TA------GA  486
P12 copy 3  CCACACATAA AAATCTTTTT TTTTTTTATG TGGTGATGGG CAAGTTCGGC GGCCCAGTAA  525

P12 copy 1  TTTAATTATT A-----TACC GATTGTCTAA AATACAGCCC TCTTTGTAAT TTTC--ATTA  538
P12 copy 2  TTTAATTATT A-----TACC GATTGTCTAA AATACAGCC- TCTTTGTAAT TTTC--ATTA  538
P12 copy 3  TATTATTATT GCTTGCTGCT GATTGCCTAA AATACAGCC- TCTTTGTAAT TCTCGCATCG  584

P12 copy 1  AATTACGAAC TACCTAGCCA TCGTGCTTTT TTTGGTCCAA ----CCAAAA AAACATATAA  594
P12 copy 2  AATTACGAAC TACCTAGCCA TCGTGCTTTT TT-GGTCCAA ----CCAAAA AA-CATTTAA  592
P12 copy 3  AATTACGAAC TACCTAGCCA TCGTGCCTTT TTTGGTCCAA AAAACCAAAA AAACGTAAAA  644

P12 copy 1  TCTAGGGG-- TTCTGCTAGC CAGCAGATAT TTTAATGATC TTT-AACTAT GATCTGAAGT  651
P12 copy 2  TCTAGGGG-- TTCTGCCAGC CAGCAGATAT TTTAATGCTC TTT-AACTAT GATCTGAAGT  649
P12 copy 3  CCTAGGGGGG TTCTGCCAGC CAGCAGATAT TTGCATGCCT TCTCAACTAT GATCTGAAGT  704
                       28S rRNA
P12 copy 1  CAAGTGGGAC TACCCGCTGA ACTT 675
P12 copy 2  CAAGTGGGAC TACCCGCTGA ACTT 673
P12 copy 3  CAAGTGGGAC TACCCGCTGA ACTT 728
```

FIG. 3. Multiple alignment of ITS sequences of *R. microsporus*. (A) Strain P11. (B) Strain P12. (C) Strain D3-1. (D) Strain D4-1. ITS1 and ITS2 are shaded in gray.

C

```
                   18S rRNA                        ITS1
D3-1 copy 1   TCCGTAGGTG AACCTGCGGA AGGATCATTA ACTAAATGTA TCGGCACTTT ACTGGGAGAG  60
D3-1 copy 2   TCCGTAGGTG AACCTGCGGA AGGATCATTA ACTAAATGTA TTGGCACTTT ACTGGGA---  57
D3-1 copy 3   TCCGTAGGTG AACCTGCGGA AGGATCATTA ACTAAATGTA TCGGCACTTT ACTGGGAGAG  60

D3-1 copy 1   GGGGGGATTC ATCCTCTCCT CCTGGTATTG TTTGCTCCTA TACTGTGAAT CTCTGGCGAT  120
D3-1 copy 2   ---------- ---TTTACTT CTCAGTATTG TTTGCTTCTA TACTGTGAAC CTCTGGCGAT  104
D3-1 copy 3   GGGGGGATTC ATCCTCTCCT CCTGGTATTG TTTGCTCCTA TACTGTGAAT CTCTGGCGAT  120

D3-1 copy 1   GAAGGTTTCG GTTGTTGTTA TGAATGATGA CTGAACCTTT GGGAGAGACT CAGGACATAT  180
D3-1 copy 2   GAAGGTC--- ---------- ------GTAA CTGA-CCTTC GGGAGAGACT CAGGACATAT  144
D3-1 copy 3   GAAGGTTTCG GTTGTTGTTA TGAATGATGA CTGAACCTTT GGGAGAGACT CAGGACATAT  180

D3-1 copy 1   AGGCTATAAT GGGTAGGCCT GTTCTGGGGT TTGATCGATG CCAATCAGGT GTGCCTTGTG  240
D3-1 copy 2   AGGCTATAAT GGGTAGGCCT GTTCTGGGGT TTGATCGATG CCAATCAGGT GTGCCTTGTG  204
D3-1 copy 3   AGGCTATAAT GGGTAGGCCT GTTCTGGGGT TTGATCGATG CCAATCAGGT GTGCCTTGTG  240

D3-1 copy 1   TACACCTGGT ACCCTTTGCC ATATACTATG AATTCAGAAT T---GAAAGT ATAATATAAT  297
D3-1 copy 2   TACACCTGGT ACCCTTTGCC ATATACTATG AATTCAGGAA TTTAAAAAGT TTAAAAAAAA  264
D3-1 copy 3   TACACCTGGT ACCCTTTGCC ATATACTATG AATTCAGGAA TTTAAAAAGT TTAAAAAAAA  300
                     5.8S rRNA
D3-1 copy 1   AA--CAACTT TTAACAATGG ATCTCTTGGT TCTCGCATCG ATGAAGAACG TAGCAAAGTG  355
D3-1 copy 2   AAAACAACTT TTAACAATGG ATCTCTTGGT TCTCGCATCG ATGAAGAACG TAGCAAAGTG  324
D3-1 copy 3   AAA-CAACTT TTAACAATGG ATCTCTTGGT TCTCGCATCG ATGAAGAACG TAGCAAAGTG  359

D3-1 copy 1   CGATAACTAG TGTGAATTGC ATATTCGTGA ATCATCGAGT CTTTGAACGC AGCTTGCACT  415
D3-1 copy 2   CGATAACTAG TGTGAATTGC ATATTCGTGA ATCATCGAGT CTTTGAACGC AGCTTGCACT  384
D3-1 copy 3   CGATAACTAG TGTGAATTGC ATATTCGTGA ATCATCGAGT CTTTGAACGC AGCTTGCACT  419
                                                         ITS2
D3-1 copy 1   CTATGGATCT TCTATAGAGT ACGCTTGCTT CAGTATCATA ACCAACCCAC ACATAAAA--  473
D3-1 copy 2   CTATGGATCT TCTATAGAGT ACGCTTGCTT CAGTATCATA ACAAACCCAC ACATAAAAAT  444
D3-1 copy 3   CTATGGATCT TCTATAGAGT ACGCTTGCTT CAGTATCATA ACAAACCCAC ACATAAAAAT  479

D3-1 copy 1   -----TTTAT TTTATGTGGT GATGGACAAG CTCGGTTA-- ----AATTTA ATTATTA---  519
D3-1 copy 2   CTTTTTTTTT TT-ATGTGGT GATGGGCAAG TTCGGCGGCC CAGTAATATT ATTATTGCTT  503
D3-1 copy 3   CTTTTTTTTT TTTATGTGGT GATGGGCAAG TTCGGCGGCC CAGTAATATT ATTATTGCTT  539

D3-1 copy 1   --TACCGGTT GTCTAAAATA CAGCCTCTTT GTAATTTTC- -ATTAAATTA CGAACTACCT  575
D3-1 copy 2   GCTGCTGATT GCCTAAAATA CAGCCTCTTT GTAATTCTCG CATCGAATTA CGAACTACCT  563
D3-1 copy 3   GCTGCTGATT GCCTAAAATA CAGCCTCTTT GTAATTCTCG CATCGAATTA CGAACTACCT  599

D3-1 copy 1   AGCCATCGTG C-TTTTTTGG TCCAA----C CAAAAAACAT ATAATCTAGG GG--TTCTGC  628
D3-1 copy 2   AGCCATCGTG CCTTTTTTGG TCCAAAAAAC CAAAAAAACG TAAACCTAGG GGGGTTCTGC  623
D3-1 copy 3   AGCCATCGTG CCTTTTTTGG TCCAAAAAAC CAAAAAAACG TAAACCTAGG GGGGTTCTGC  659
                                                                      28S rRNA
D3-1 copy 1   TAGCCAGCAG ATATTTTAAT GATCTTT-AA CTATGATCTG AAGTCAAGTG GGACTACCCG  687
D3-1 copy 2   CAGCCAGCAG ATATTTGCAT GCCTTCTCAA CTATGATCTG AAGTCAAGTG GGACTACCCG  683
D3-1 copy 3   CAGCCAGCAG ATATTTGCAT GCCTTCTCAA CTATGATCTG AAGTCAAGTG GGACTACCCG  719

D3-1 copy 1   CTGA 691
D3-1 copy 2   CTGA 687
D3-1 copy 3   CTGA 723
```

FIG. 3—*Continued.*

MATERIALS AND METHODS

Strains. The four strains of *R. microsporus* used in this study were isolated from two patients (P11 and P12) and two tablets of allopurinol (D3-1 and D4-1) during the outbreak investigation of intestinal mucormycosis in patients with hematological malignancies in Hong Kong (5). All four strains were identified to be *R. microsporus* by their morphological appearance and scanning electron microscopy (5).

DNA extraction. Fungal DNA extraction was performed as described in our previous publications (24, 26). Briefly, DNA was extracted from 1 g of fungal cells in 10 ml of distilled water using a DNeasy plant minikit according to the manufacturer's instructions (Qiagen, Hilden, Germany). The extracted DNA was eluted in 50 μl of kit buffer AE, the resultant mixture was diluted 10 times, and 1 μl of the diluted extract was used for PCR.

PCR, gel electrophoresis, and ITS sequencing. PCR amplification and DNA sequencing of the ITS regions of the four strains of *R. microsporus* were performed according to published protocols (23, 27). Briefly, DNase I-treated distilled water and PCR Master Mix (which contains deoxynucleoside triphosphates [dNTPs], PCR buffer, and *Taq* polymerase) were used in all PCRs by adding 1 U of DNase I (Pharmacia, Sweden) to 40 μl of distilled water or PCR Master Mix and incubating the mixture at 25°C for 15 min and subsequently at 95°C for 10 min to inactivate the DNase I. The fungal DNA extract and controls were amplified with 0.5 μM primers (ITS1, 5′-TCCGTAGGTGAACCTGCGG-3′; ITS4, 5′-TCCTCCGCTTATTGATATGC-3′) (Gibco BRL, Rockville, MD). The PCR mixture (25 μl) contained fungal DNA, PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, and 0.01% gelatin), 200 μM each dNTP, and 1.0 U of *Taq* polymerase (Applied Biosystems, Foster City, CA). The mixtures were amplified in 40 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, with a final extension at 72°C for 10 min in an automated thermal cycler (Applied Biosystem, Foster City, CA). *R. microsporus* strain P2 was used as the positive control, and DNase I-treated distilled water was the negative control (5). Ten microliters of each amplified product was electrophoresed in 1.5% (wt/vol) agarose gel, with a molecular size marker (Lambda AvaII digest; Fermentas, Ontario, Canada) in parallel. Electrophoresis in Tris-borate-EDTA buffer was performed at 100 V for 1.5 h. The gel was stained with ethidium bromide (0.5 μg/ml) for 15 min, rinsed, and photographed under UV light illumination.

The PCR products were gel purified using a QIAquick PCR purification kit (QIAgen, Hilden, Germany). Both strands of the purified PCR product for each strain were sequenced twice with an ABI Prism 3700 DNA Analyzer (Applied Biosystems, Foster City, CA) and using the PCR primers ITS1 and ITS4. In addition, the purified PCR products were also cloned into the pT-Adv vector (BD Biosciences) according to the manufacturer's instructions. Both strands of 10 of the clones for each strain were sequenced twice, using primers ITS1 and ITS4. The sequences of the cloned PCR products were compared with known ITS gene sequences of closely related species in the GenBank by multiple sequence alignment using ClustalX, version 1.83 (20).

Phylogenetic characterization. Phylogenetic tree construction was performed using the neighbor-joining method with ClustalX, version 1.83. The trees were constructed by the neighbor-joining method using a Jukes-Cantor correction. A total of 737 nucleotide positions were included in the analysis.

Nucleotide sequence accession numbers. The ITS sequences of the four strains of *R. microsporus* have been deposited in the GenBank under accession numbers GQ502275 to GQ502285.

RESULTS

Direct ITS sequencing. PCR of the ITS regions of the four *R. microsporus* strains, P11, P12, D3-1, and D4-1, showed thick bands at about 700 bp (Fig. 1). Direct sequencing of the puri-

```
D
             18S rRNA                          ITS1
D4 copy1   TCCGTAGGTG AACCTGCGGA AGGATCATTA ACTAA-TGTA TTGGCACTTT ACTGGGA---   56
D4 copy2   TCCGTAGGTG AACCTGCGGA AGGATCATTA ACTAA-TGTA TTGGCACTTT ACTGGGA---   56
D4 copy3   TCCGTAGGTG AACCTGCGGA AGGATCATTA ACTAAATGTA TCGGCACTTT ACTGGGAGAG   60

D4 copy1   ---------- ---TTTACTT CTCAGTATTG TTTGCT-CTA TACTGTGAAC CTCTGGCGAT  102
D4 copy2   ---------- ---TTTACTT CTCAGTATTG TTTGCTTCTA TACTGTGAAC CTCTGGCGAT  103
D4 copy3   GGGGGGATTC ATCCTCTCCT CCTGGTATTG TTTGCTCCTA TACTGTGAAT CTCTGGCGAT  120

D4 copy1   GAAGGTC--- ---------- ------GTAA CTGA-CCTTC GGGAGAGACT CAGGACATAT  142
D4 copy2   GAAGGTC--- ---------- ------GTAA CTGA-CCTTC GGGAGAGACT CAGGACATAT  143
D4 copy3   GAAGGTTTCG GTTGTTGTTA TGAATGATGA CTGAACCTTT GGGAGAGACT CAGGACATAT  180

D4 copy1   AGGCTATAAT GGGTAGGCCT GTTCTGGGGT TTGATCGATG CCAATCAGGA TTACCTTTCT  202
D4 copy2   AGGCTATAAT GGGTAGGCCT GTTCTGGGGT TTGATCGATG CCAATCAGGA TTACCTTTCT  203
D4 copy3   AGGCTATAAT GGGTAGGCCT GTTCTGGGGT TTGATCGATG CCAATCAGGT GTGCCTTGTG  240

D4 copy1   TCCTTTGGGA AGGAAGGTGC CTGGTACCCT TTACCATATA CCATGAATTC AG-AATTGAA  261
D4 copy2   TCCTTTGGGA AGGAAGGTGC CTGGTACCCT TTACCATATA CCATGAATTC AG-AATTGAA  262
D4 copy3   TAC------- --------AC CTGGTACCCT TTGCCATATA CTATGAATTC AGGAATTTAA  285
                                  5.8S rRNA
D4 copy1   AGTAT---AA TATAATAACA ACTTTTAACA ATGGATCTCT TGGTTCTCGC ATCGATGAAG  318
D4 copy2   AGTAT---AA TATAATAACA ACTTTTAACA ATGGATCTCT TGGTTCTCGC ATCGATGAAG  319
D4 copy3   AAAGTTTAAA AAAAAAAACA ACTTTTAACA ATGGATCTCT TGGTTCTCGC ATCGATGAAG  345

D4 copy1   AACGTAGCAA AGTGCGATAA CTAGTGTGAA TTGCATATTC GTGAATCATC GAGTCTTTGA  378
D4 copy2   AACGTAGCAA AGTGCGATAA CTAGTGTGAA TTGCATATTC GTGAATCATC GAGTCTTTGA  379
D4 copy3   AACGTAGCAA AGTGCGATAA CTAGTGTGAA TTGCATATTC GTGAATCATC GAGTCTTTGA  405
                                                                    ITS2
D4 copy1   ACGCAGCTTG CACTCTATGG ATCTTCTATA GAGTACGCTT GCTTCAGTAT CATAACCAAC  438
D4 copy2   ACGCAGCTTG CACTCTATGG ATCTTCTATA GAGTACGCTT GCTTCAGTAT CATAACCAAC  439
D4 copy3   ACGCAGCTTG CACTCTATGG ATCTTCTATA GAGTACGCTT GCTTCAGTAT CATAACAAAC  465

D4 copy1   CCACACATAA AA-------T TTATTTTATG TGGTGATGGA CAAGCTCGGT TA------AA  485
D4 copy2   CCACACATAA AA-------T TTATTTTATG TGGTGATGGA CAAGCTCGGT TA------AA  486
D4 copy3   CCACACATAA AAATCTTTTT TTTTTTTATG TGGTGATGGG CAAGTTCGGC GGCCCAGTAA  525

D4 copy1   TTTAATTATT A-----TACC GATTGTCTAA AATACAGCCC TCTTTGTAAT TTTC--ATTA  538
D4 copy2   TTTAATTATT A-----TACC GATTGTCTAA AATACAGCC- TCTTTGTAAT TTTC--ATTA  538
D4 copy3   TATTATTATT GCTTGCTGCT GATTGCCTAA AATACAGCC- TCTTTGTAAT TCTCGCATCG  584

D4 copy1   AATTACGAAC TACCTAGCCA TCGTGCTTTT TTTGGTCCAA ----CCAAAA AAACATATAA  594
D4 copy2   AATTACGAAC TACCTAGCCA TCGTGCTTTT TT-GGTCCAA ----CCAAAA AA-CATATAA  592
D4 copy3   AATTACGAAC TACCTAGCCA TCGTGCCTTT TTTGGTCCAA AAAACCAAAA AAACGTAAAA  644

D4 copy1   TCTAGGGG-- TTCTGCTAGC CAGCAGATAT TTTAATGATC TTT-AACTAT GATCTGAAGT  651
D4 copy2   TCTAGGGG-- TTCTGCTAGC CAGCAGATAT TTTAATGATC TTT-AACTAT GATCTGAAGT  649
D4 copy3   CCTAGGGGGG TTCTGCCAGC CAGCAGATAT TTGCATGCCT TCTCAACTAT GATCTGAAGT  704
                      28S rRNA
D4 copy1   CAAGTGGGAC TACCCGCTGA ACTT 675
D4 copy2   CAAGTGGGAC TACCCGCTGA ACTT 673
D4 copy3   CAAGTGGGAC TACCCGCTGA ACTT 728
```

FIG. 3—*Continued.*

fied bands showed double peaks frequently along all of the sequence traces (Fig. 2). For the sequence traces of P12 D3-1 and D4-1, occasional triple peaks were also observed (Fig. 2B, C, and D).

Sequencing of cloned PCR products. The thick bands of the four *R. microsporus* strains were purified and cloned into the pT-Adv vector. Sequencing of 10 clones for each strain revealed two different ITS sequences for strain P11 (Fig. 3A) and three different ITS sequences for strains P12, D3-1, and D4-1 (Fig. 3B, C, and D). For strain P11, 2 of the 10 sequences were of one type (Fig. 3A, copy 1) and 8 were of a second type (Fig. 3B, copy 2); for strain P12, 5 of the 10 sequences were of one type (Fig. 3B, copy 1), 4 were of a second type (Fig. 3B, copy 2), and 1 was of a third type (Fig. 3B, copy 3); for strain D3-1, 3 of the 10 sequences were of one type (Fig. 3C, copy 1), 3 were of a second type (Fig. 3C, copy 2), and 4 were of a third type (Fig. 3C, copy 3); and for strain D4-1, 6 of the 10 sequences were of one type (Fig. 3D, copy 1), 2 were of a second type (Fig. 3D, copy 2), and 2 were of a third type (Fig. 3D, copy 3). For strain P11, there were 141 (19.1%) nucleotide differences between copies 1 and 2 (Fig. 3A). For strain P12, there were 13 (1.7%) nucleotide differences between copies 1 and 2, 142 (19.2%) nucleotide differences between copies 1 and 3, and 144 (19.4%) nucleotide differences between copies 2 and 3 (Fig. 3B). For strain D3-1, there were 121 (16.7%) nucleotide differences between copies 1 and 2, 69 (9.5%) nucleotide differences between copies 1 and 3, and 52 (7.2%) nucleotide differences between copies 2 and 3 (Fig. 3C). For strain D4-1, there were 4 (0.6%) nucleotide differences between copies 1 and 2, 142 (19.1%) nucleotide differences between copies 1 and 3, and 143 (19.2%) nucleotide differences between copies 2 and 3 (Fig. 3D). The sequence of copy 2 of strain P11 was identical to sequences of copy 1 of strain P12 and copy 1 of D4-1, and the sequence of copy 1 of strain P11 was identical to that of copy 3 of strain P12, copy 3 of strain D3-1, and copy 3 of strain D4-1 (Fig. 4).

DISCUSSION

We report the first evidence of ITS sequence heterogeneity in *Mucorales*. In this study and the recent outbreak investigation of intestinal *R. microsporus* infections (5), four (14%) of the 28 *R. microsporus* strains isolated were found to possess ITS sequence heterogeneity. Since a major component of the

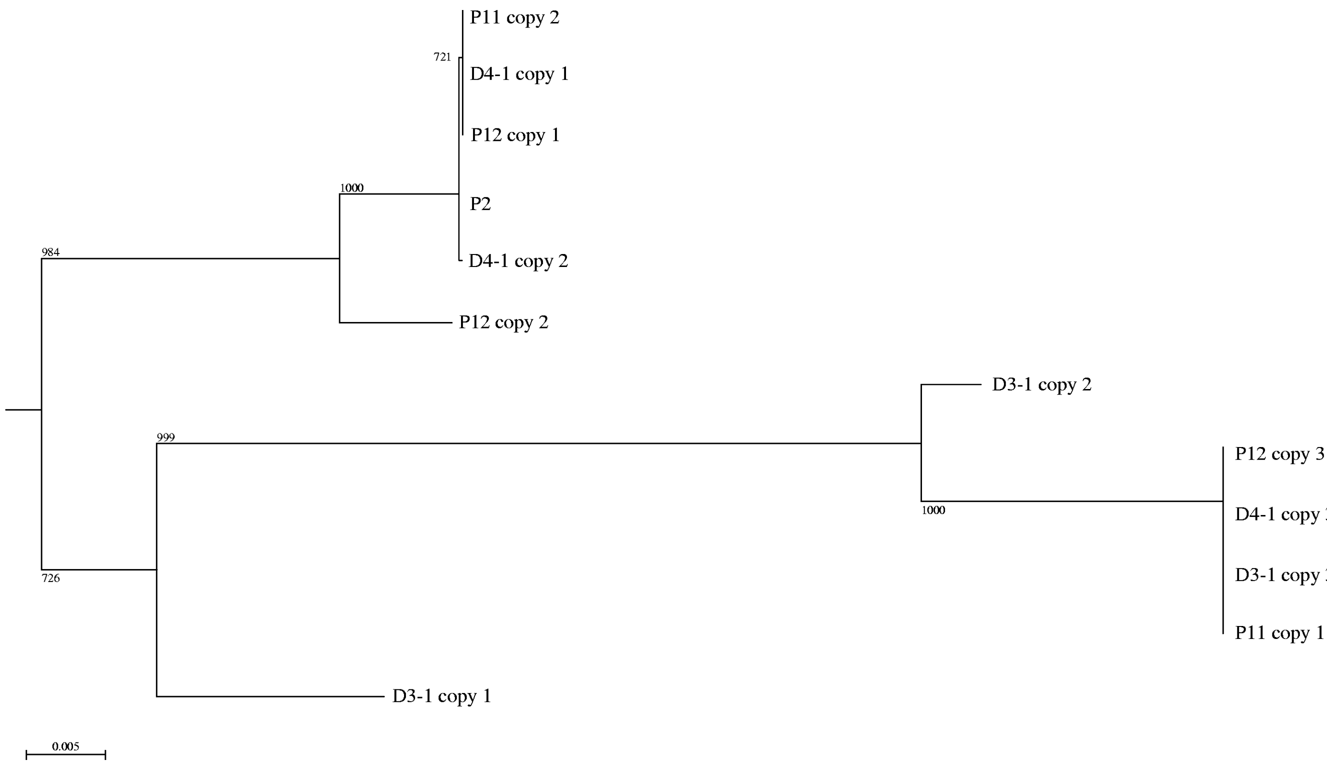

FIG. 4. Phylogenetic tree showing the relationship of the four strains of *R. microsporus*. The tree was inferred from ITS sequence data (737 nucleotide positions) by the neighbor-joining method and was rooted using *Absidia blakesleeana* (AY944894). The scale bar indicates the estimated number of substitutions per 200 bases. Numbers at nodes indicate levels of bootstrap support calculated from 1,000 trees.

heterogeneous nature of the ITS was due to DNA insertion/deletion, small differences in the lengths of the PCR products were generated during amplification of the ITS regions of the *R. microsporus* strains. This gave rise to the thick bands observed in agarose gel electrophoresis (Fig. 1). Furthermore, double and occasionally triple peaks were observed when the PCR products were directly sequenced because two or more kinds of PCR products were sequenced simultaneously (Fig. 2). Cloning the PCR products and sequencing 10 clones from each of the four *R. microsporus* strains confirmed ITS sequence heterogeneity in all four *R. microsporus* strains isolated. This is different from the observation described in *Pneumocystis jiroveci*, for which direct PCR sequencing of its ITS regions in clinical samples showed heterogeneous sequences, which probably represent different strains of *P. jiroveci* infecting the same patient instead of different ITS sequences in the same strain (8).

Variations in ITS sequence among the different rDNA operons in the same strain of *R. microsporus* were observed only in ITS1 and ITS2 but not the 5.8S rDNA region. The mature 28S, 5.8S, and 5S rRNA, assembled with the many ribosomal proteins, form the larger subunit of the ribosome in eukaryotes. Since the 5.8S rRNA is an essential functional component of only approximately 160 nucleotides in length, minimal variations in its sequence among the different rDNA operons is expected. In fact, its sequence is relatively conserved among fungi of different species. On the other hand, for ITS1 and ITS2, although they have a role in the development of functional RNA, sequence variations among fungi of different species and even among different strains of the same fungal species are much more common. This phenomenon is also present in the four strains of *R. microsporus* with ITS sequence heterogeneity in the present study. As shown in Fig. 3, all the sequence variations were observed in ITS1 and ITS2 for all four strains of *R. microsporus.* It is notable that copy 2 of strain P11, copy 1 of strain P12, and copy 1 of strain D4-1 showed identical sequences; and copy 1 of strain P11, copy 3 of strain P12, copy 3 of strain D3-1, and copy 3 of strain D4-1 also showed identical sequences (Fig. 4). This implies that hot spots of insertion/deletion in ITS1 and ITS2 may be present.

ITS sequence heterogeneity is an obstacle to molecular identification and genotyping of pathogenic fungi in clinical microbiology laboratories. Accurate identification of pathogenic fungi is the cornerstone to prescribing antifungal treatment (2, 7, 9, 17); for example, identification of *R. microsporus* will necessitate the prescription of a combination treatment of posaconazole, amphotericin B, and caspofungin as posaconazole has been shown to have synergistic effects with amphotericin B and caspofungin against the *Mucorales* (6, 16). For molecular identification of fungal pathogens, the ITS is one of the most commonly used targets because its length and sequence are relatively conserved for the same fungal species but often different in different fungal species (4, 23). On the other hand, due to variation in ITS sequence among different strains in some fungal species, it has also been used for fungal genotyping (11, 14, 21). For example, in the recent outbreak of intestinal mucormycosis, eight alleles were observed among 24 strains of *R. microsporus*, thereby confirming multiple strain involvement in the outbreak (5). This number of different alleles was large compared to that for the gene loci used in multilocus sequence typing schemes (MLST) of molds. For example, in our recently published highly discriminatory MLST scheme for *Penicillium marneffei*, only 5 to 11 alleles were observed among 44 strains of *P. marneffei* for each of the five individual gene loci despite the very high evolutionary rates of all the five gene loci (24). Not only is the cloning of PCR fragments labor-intensive and time-consuming, but the technology is also often not available in most clinical microbiology laboratories. Therefore, variations of ITS sequences in different rDNA operons within the same strain of fungus will make identification and typing of such a strain by ITS sequencing very difficult in clinical microbiology laboratories. When thick bands and double peaks are observed during PCR sequencing of a gene target, such a strain should be sent to reference laboratories proficient in molecular technologies for further identification and/or genotyping.

ACKNOWLEDGMENTS

This work is partly supported by the Consultancy Service for Enhancing Laboratory Surveillance of Emerging Infectious Disease for the Department of Health of the Hong Kong Special Administrative Region of China, a Research Grant Council Grant, the University Development Fund, a Committee for Research and Conference grant, and an Outstanding Young Researcher Award from The University of Hong Kong.

REFERENCES

1. **Almyroudis, N. G., D. A. Sutton, P. Linden, M. G. Rinaldi, J. Fung, and S. Kusne.** 2006. Zygomycosis in solid organ transplant recipients in a tertiary transplant center and review of the literature. Am. J. Transplant. **6:**2365–2374.
2. **Alvarez, E., D. A. Sutton, J. Cano, A. W. Fothergill, A. Stchigel, M. G. Rinaldi, and J. Guarro.** 2009. Spectrum of zygomycete species identified in clinically significant specimens in the United States. J. Clin. Microbiol. **47:**1650–1656.
3. **Amann, G., K. O. Stetter, E. Llobet-Brossa, R. Amann, and J. Anton.** 2000. Direct proof for the presence and expression of two 5% different 16S rRNA genes in individual cells of *Haloarcula marismortui*. Extremophiles **4:**373–376.
4. **Chen, Y. C., J. D. Eisner, M. M. Kattar, S. L. Rassoulian-Barrett, K. Lafe, U. Bui, A. P. Limaye, and B. T. Cookson.** 2001. Polymorphic internal transcribed spacer region 1 DNA sequences identify medically important yeasts. J. Clin. Microbiol. **39:**4042–4051.
5. **Cheng, V. C., J. F. Chan, A. H. Ngan, K. K. To, S. Y. Leung, H. W. Tsoi, W. C. Yam, J. W. Tai, S. S. Wong, H. Tse, I. W. Li, S. K. Lau, P. C. Woo, A. Y. Leung, A. K. Lie, R. H. Liang, T. L. Que, P. L. Ho, and K. Y. Yuen.** 2009. Outbreak of intestinal infection due to *Rhizopus microsporus*. J. Clin. Microbiol. **47:**2834–2843.
6. **Guembe, M., J. Guinea, T. Peláez, M. Torres-Narbona, and E. Bouza.** 2007. Synergistic effect of posaconazole and caspofungin against clinical zygomycetes. Antimicrob. Agents Chemother. **51:**3457–3458.
7. **Hata, D. J., S. P. Buckwalter, B. S. Pritt, G. D. Roberts, and N. L. Wengenack.** 2008. Real-time PCR method for detection of zygomycetes. J. Clin. Microbiol. **46:**2353–2358.
8. **Helweg-Larsen, J., B. Lundgren, and J. D. Lundgren.** 2001. Heterogeneity and compartmentalization of *Pneumocystis carinii* f. sp. *hominis* genotypes in autopsy lungs. J. Clin. Microbiol. **39:**3789–3792.
9. **Johnson, E. M., A. Szekely, and D. W. Warnock.** 1998. In vitro activity of voriconazole, itraconazole and amphotericin B against filamentous fungi. J. Antimicrob. Chemother. **42:**741–745.
10. **Lau, S. K., P. C. Woo, S. K. Chiu, K. W. Leung, R. W. Yung, and K. Y. Yuen.** 2003. Early diagnosis of *Exophiala* CAPD peritonitis by 18S ribosomal RNA gene sequencing and its clinical significance. Diagn. Microbiol. Infect. Dis. **46:**95–102.
11. **McManus, B. A., D. C. Coleman, G. Moran, E. Pinjon, D. Diogo, M. E. Bougnoux, S. Borecká-Melkusova, H. Bujdákova, P. Murphy, C. d'Enfert, and D. J. Sullivan.** 2008. Multilocus sequence typing reveals that the population structure of *Candida dubliniensis* is significantly less divergent than that of *Candida albicans*. J. Clin. Microbiol. **46:**652–664.
12. **Nubel, U., B. Engelen, A. Felske, J. Snaidr, A. Wieshuber, R. I. Amann, W. Ludwig, and H. Backhaus.** 1996. Sequence heterogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis. J. Bacteriol. **178:**5636–5643.
13. **O'Donnell, K., and E. Cigelnik.** 1997. Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. Mol. Phylogenet. Evol. **7:**103–116.
14. **O'Donnell, K., D. A. Sutton, A. Fothergill, D. McCarthy, M. G. Rinaldi, M. E. Brandt, N. Zhang, and D. M. Geiser.** 2008. Molecular phylogenetic diversity, multilocus haplotype nomenclature, and in vitro antifungal resistance within the *Fusarium solani* species complex. J. Clin. Microbiol. **46:**2477–2490.
15. **Okabe, I., M. Arakawa, and N. Matsumoto.** 2001. ITS polymorphism within a single strain of *Sclerotium rolfsii*. Mycoscience **42:**107–113.
16. **Perkhofer, S., M. Locher, M. Cuenca-Estrella, R. Rüchel, R. Würzner, M. P. Dierich, and C. Lass-Flörl.** 2008. Posaconazole enhances the activity of amphotericin B against hyphae of zygomycetes in vitro. Antimicrob. Agents Chemother. **52:**2636–2638.
17. **Pfaller, M. A., and D. J. Diekema.** 2004. Rare and emerging opportunistic fungal pathogens: concern for resistance beyond *Candida albicans* and *Aspergillus fumigatus*. J. Clin. Microbiol. **42:**4419–4431.
18. **Sims, C. R., and L. Ostrosky-Zeichner.** 2007. Contemporary treatment and outcomes of zygomycosis in a non-oncologic tertiary care center. Arch. Med. Res. **38:**90–93.
19. **Siwek, G. T., K. J. Dodgson, M. de Magalhaes-Silverman, L. A. Bartelt, S. B. Kilborn, P. L. Hoth, D. J. Diekema, and M. A. Pfaller.** 2004. Invasive zygomycosis in hematopoietic stem cell transplant recipients receiving voriconazole prophylaxis. Clin. Infect. Dis. **39:**584–587.
20. **Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins.** 1997. The CLUSTAL_X Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. **25:**4876–4882.
21. **van Hal, S. J., F. Gilgado, T. Doyle, J. Barratt, D. Stark, W. Meyer, and J. Harkness.** 2009. Clinical significance and phylogenetic relationship of novel Australian *Pneumocystis jirovecii* genotypes. J. Clin. Microbiol. **47:**1818–1823.
22. **Wang, D. M., and Y. J. Yao.** 2005. Intrastrain internal transcribed spacer heterogeneity in *Ganoderma* species. Can. J. Microbiol. **51:**113–121.
23. **White, T. J., T. Bruns, S. Lee, and J. W. Taylor.** 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, p. 315–322. *In* M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (ed.), PCR protocols: a guide to methods and applications. Academic Press, Inc., New York, NY.
24. **Woo, P. C., C. C. Lau, K. T. Chong, H. Tse, D. N. Tsang, R. A. Lee, C. W. Tse, T. L. Que, L. M. Chung, A. H. Ngan, W. T. Hui, S. S. Wong, S. K. Lau, and K. Y. Yuen.** 2007. *MP1* homologue-based multilocus sequence typing system for typing the pathogenic fungus *Penicillium marneffei*: a novel approach using lineage-specific genes. J. Clin. Microbiol. **45:**3647–3654.
25. **Woo, P. C., J. L. Teng, K. W. Leung, S. K. Lau, G. K. Woo, A. C. Wong, M. K. Wong, and K. Y. Yuen.** 2005. *Anaerospora hongkongensis* gen. nov. sp. nov., a novel genus and species with ribosomal DNA operon heterogeneity isolated from an intravenous drug abuser with pseudobacteraemia. Microbiol. Immunol. **49:**31–39.
26. **Woo, P. C., K. T. Chong, H. Tse, J. J. Cai, C. C. Lau, A. C. Zhou, S. K. Lau, and K. Y. Yuen.** 2006. Genomic and experimental evidence for a potential sexual cycle in the pathogenic thermal dimorphic fungus *Penicillium marneffei*. FEBS Lett. **580:**3409–3416.
27. **Woo, P. C., S. K. Lau, A. H. Ngan, H. Tse, E. T. Tung, and K. Y. Yuen.** 2008. *Lasiodiplodia theobromae* pneumonia in a liver transplant recipient. J. Clin. Microbiol. **46:**380–384.
28. **Woo, P. C., S. K. Lau, J. L. Teng, H. Tse, and K. Y. Yuen.** 2008. Then and now: use of 16S rDNA gene sequencing for bacterial identification and discovery of novel bacteria in clinical microbiology laboratories. Clin. Microbiol. Infect. **14:**908–934.